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***Schistosoma haematobium* Egg-Induced Bladder Urothelial Abnormalities Dependent on *p53* are Modulated by Host Sex**

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Abstract

Introduction and Objective—The bladder urothelium changes dramatically during *Schistosoma haematobium* infection (urogenital schistosomiasis). These alterations include hyperplasia, ulceration, dysplasia, squamous metaplasia and frank carcinogenesis. Defining the pathways underpinning these urothelial responses will contribute to a deeper understanding of how *S. haematobium* egg expulsion, hematuria, and bladder cancer develop in humans. The tumor suppressor gene *p53* is of particular interest, given its role in many cancers, including bladder cancer generally and schistosomal bladder cancer specifically.

Methods—Transgenic mice featuring tamoxifen-inducible *Cre* recombinase activity in cells expressing the urothelial-specific gene *uroplakin-3a* (*Upk3a*-GCE mice) were crossed with either TdTomato-floxed-EGFP reporter or *p53*-floxed mice. Mice were administered tamoxifen or vehicle control to induce excision of floxed genes. TdTomato-EGFP reporter mice were sacrificed and their bladders harvested, sectioned, and imaged by fluorescence microscopy. *p53*-floxed mice underwent bladder wall injection with *S. haematobium* eggs or vehicle controls. Three months later, mice were sacrificed and their bladders subjected to histological analysis (H&E staining).

Results—We first confirmed the phenotypic fidelity of *Upk3a*-GCE mice by crossing them with TdTomato-floxed-EGFP reporter mice and administering tamoxifen to their progeny. As expected, these progeny switched from TdTomato to EGFP expression in their bladder urothelium. Having confirmed the phenotype of *Upk3a*-GCE mice, we next crossed them to *p53*-floxed mice. The resulting progeny were given tamoxifen or vehicle control to render them urothelial *p53*-haploinsufficient or –intact, respectively. Then, we injected *S. haematobium* eggs or control vehicle into the bladder walls of these mice. Male *p53*-intact, egg-injected mice exhibited similar

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histological changes as their *p53*-haploinsufficient counterparts, including urothelial hyperplasia and ulceration. In contrast, female *p53*-intact, egg-injected mice featured no urothelial ulceration, whereas their *p53*-haploinsufficient counterparts often had significant ulceration.

Conclusions—Urothelial *p53* signaling indeed seems to affect urothelial homeostasis during *S. haematobium* infection, albeit in a sex-specific manner. Ongoing work seeks to determine whether *p53* mediates associated alterations in urothelial cell cycle status and frank carcinogenesis in the setting of urogenital schistosomiasis.

Keywords

bladder cancer; schistosomiasis; schistosoma; schistosoma haematobium; haematobium; bilharzia; *p53*; ulceration; urothelium; urothelial

Introduction

Schistosomiasis is a chronic infection caused by parasitic *Schistosoma* worms, with up to 200 million people requiring drug treatment annually. *Schistosoma haematobium*, the most prevalent species globally, is a well-recognized risk factor for the development of bladder cancer. Eggs continually laid by *S. haematobium* adult worm pairs sustain chronic granulomatous inflammation in the bladder walls of humans, causing severe pathology that can lead to the development of bladder cancer in later life. The mechanisms by which this prolonged inflammation promotes bladder cancer are poorly understood, due substantially to the lack of an animal model recapitulating human infection and bladder pathology.

We have developed a technique of surgically injecting a bolus of *S. haematobium* eggs into the bladder walls of mice that recapitulates the chronic granulomatous inflammation seen in human infections (Fu et al., 2012). The cytokine environment is definitively type-2, with prolonged IL-4 upregulation in both the bladder and regional lymph nodes.

Current bladder cancer models in rodents rely on the administration of nitrosamines in drinking water, leading to onset of invasive carcinoma after 3–4 months of exposure. These tumors will develop without prior inflammation in the bladder, and the relatively high dose of nitrosamines used may not represent the normal conditions of schistosomal bladder cancer development. Given the known role of *p53* mutations in the progression of bladder cancers, including schistosomal bladder cancer, we sought to genetically perturb *p53* levels in bladder urothelium and look for evidence of early events in transformation of urothelial cells to carcinoma *in situ* after egg injection.

Methods

Ethics Statement

All animal work was conducted according to relevant U.S. and international guidelines. Specifically, all experimental procedures were carried out in accordance with the Administrative Panel on Laboratory Animal Care (APLAC) protocol and the institutional guidelines set by the Veterinary Service Center at Stanford University (Animal Welfare Assurance A3213-01 and USDA License 93-4R-00). Stanford APLAC and institutional

guidelines are in compliance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Stanford APLAC approved the animal protocol associated with the work described in this publication.

Mice

B6;DBA-Tg(*Upk3a*-GFP/cre/ERT2)26Amc/J mice (hereafter referred to as *Upk3a*-GCE mice), feature tamoxifen-inducible Cre recombinase activity in cells expressing uroplakin-3a, a urothelial-specific gene (Harding et al., 2011). These mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and crossed with either TdTomato-floxed-EGFP reporter (*Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}/J*) or *p53*-floxed mice obtained from Jackson Laboratories or as a gift from Zijie Sun, respectively.

Mouse procedures and microscopy

Mice were injected intraperitoneally with 3 consecutive daily doses of 0.13g/kg tamoxifen in corn oil or corn oil alone (Sigma) to induce excision of floxed genes. *Upk3a*-GCE^{+/WT};TdTomato-EGFP^{+/WT} reporter mice were sacrificed 5 days later, with bladders embedded and snap frozen in OCT. The tissues were sectioned onto glass slides and briefly air-dried, followed by mounting in ProLong Gold (Invitrogen) for imaging of native fluorescence by microscopy. Five days after the final tamoxifen injection, *Upk3a*-GCE^{+/WT};p53^{flox/WT} mice underwent bladder wall injection with 3000 *S. haematobium* eggs or vehicle controls as previously described (Fu et al., 2012, 2011). Three months after egg injection, mice were sacrificed and bladders were formalin-fixed, embedded and sectioned for H&E staining. These bladder tissues were evaluated in a blinded manner by an experienced urologic pathologist (OH).

Results

Confirmation of urothelial specificity of Cre activity in *Upk3a*-GCE mice

We first sought to confirm that Cre activity in *Upk3a*-GCE mice was specific for the bladder urothelium and not other bladder tissues. *Upk3a*-GCE mice were crossed to TdTomato-EGFP reporter mice (*mTmG*). The F1 progeny (*Upk3a*-GCE^{+/WT};mTmG^{+/WT}) were administered tamoxifen to induce Cre-mediated excision of the floxed, membrane-targeted TdTomato cassette (*mT*), allowing expression of the downstream membrane-targeted EGFP (*mG*) cassette. As anticipated, this resulted in selective EGFP expression in the bladder urothelium, verifying that Cre activity in *Upk3a*-GCE mice was selectively induced in this epithelial tissue but not elsewhere in the bladder (Figure 1).

Rendering mouse bladder urothelium *p53*-haploinsufficient

After confirming the urothelial specificity of Cre activity in *Upk3a*-GCE mice, we bred these animals to *p53*^{flox/flox} mice. Administration of tamoxifen to F1 progeny (*Upk3a*-GCE^{+/WT};p53^{flox/WT}) yielded bladder urothelial cells with only one functioning *p53* allele (*p53*^{+/WT}). Bladder wall injection of hamster-tissue derived *S. haematobium* eggs or hamster tissue extract 5 days after the final tamoxifen dose induced bladder alterations that were assessed by H&E staining of formalin-fixed, paraffin-embedded tissue 12 weeks later.

***S. haematobium* egg-induced effects on the *p53*-haploinsufficient urothelium depend on host sex**

Male mice, with or without full *p53* activity in the urothelium, exhibited similar histological features at 12 weeks after egg-injection (Figure 2 and Table 1). Female egg-injected mice without full *p53* activity showed increased rates of ulceration, and egg-injected female mice generally exhibited more regions of urothelial squamous metaplasia (Figure 3 and Table 1). Interestingly, the extent of dysplasia was generally more severe in female egg-injected, tamoxifen-treated (*p53*-haploinsufficient) animals (Figure 4 and Table 1). Urothelial regions distant from any underlying granuloma harbored more hyperplastic and dysplastic features, potentially related to early shedding of umbrella cells overlying the egg injection site.

Discussion

Urogenital schistosomiasis, infection by *S. haematobium*, often results in egg deposition in the host bladder. In this environment *S. haematobium* eggs are highly inflammatory, triggering granuloma formation and a range of urothelial abnormalities, including urothelial hyperplasia (with or without nuclear atypia), ulceration, sloughing, squamous metaplasia, formation of polyploid lesions, dysplasia, carcinoma *in situ*, and muscle-invasive urothelial cell carcinoma, squamous cell carcinoma, and adenocarcinoma (Khafagy et al., 1972; Sabe et al., 2008; Smith et al., 1977). In this study we used our mouse model of *S. haematobium* egg-induced bladder pathology (Fu et al., 2012). Not surprisingly, granuloma development and urothelial hyperplasia were essentially universal findings in egg-exposed bladders, recapitulating similar features in human urogenital schistosomiasis.

Numerous publications have implicated *p53* abnormalities in schistosomal bladder carcinogenesis (Cooper et al., 1997; Helal et al., 2006; Khaled et al., 2003; Osman et al., 1997; Santos et al., 2014). *p53* additionally plays key roles at cell cycle checkpoints for tissues throughout the body, where it halts cell cycle progression in the face of regenerative signaling associated with cellular damage (Enoch and Norbury, 1995). Accordingly, we found that urothelial haploinsufficiency of *p53* led to increased ulceration following *S. haematobium* egg injection. This *p53* deficiency-associated urothelial ulceration appeared to be a more frequent event in female mice. This was surprising to us, given that urothelial ulceration can be a sign of bladder malignancy (“Bladder: Urothelial carcinomas,” n.d.), and bladder cancer is more common in men than women in industrialized countries (Madeb and Messing, 2004). A higher incidence of schistosomal bladder cancer in males has been observed in certain regions, though this sex bias may be due to differences in exposure to infection (Mostafa et al., 1999). Though bladder cancer is generally of lower incidence in females, oncologic outcomes may be worse for females compared to men at a similar tumor stage (Mungan et al., 2000); this may be partly attributed to delays in diagnosis compared to males (Cohn et al., 2014), or potentially differences in sex hormone receptor expression of tumor tissue, and may also depend on the patient population studied (Cantwell et al., 2006; McGrath et al., 2006).

The increased ulceration we observe in *p53*-haploinsufficient female mice injected with *Schistosoma haematobium* eggs occurs in the pre-neoplastic stage. This increase in ulceration is not likely due to pronounced off-target effects of tamoxifen on the urothelium

of female mice compared to males, as both male and female mice given tamoxifen followed by control (hamster-extract) injection of the bladder exhibited some hyperplasia, and signs of atypia with hyperplasia in this experimental group were in fact more pronounced in males than in females (Table 1). This suggests an interaction between sex and observed urothelial abnormalities in the context of inflammation caused by *Schistosoma haematobium* infection. Of note, we observed urothelial dysplasia in some egg-injected *p53*-haploinsufficient mice, which was suggestive of pre-neoplasia, but never outright carcinogenesis. Likewise, although we noted the presence of squamous metaplasia in the urothelia of a number of *p53*-haploinsufficient mice, we did not see any squamous cell carcinomas. We postulate that additional “hits” besides *S. haematobium* egg exposure and *p53* haploinsufficiency may be necessary to trigger frank carcinogenesis in the mammalian bladder, which is consistent with Knudson’s multi-hit hypothesis of cancer (Knudson, 1971). Possible risk factors that may lead to further mutational hits include carcinogen exposure, i.e., nitrosamines created by bacterial uropathogens or present in food or tobacco products (Hicks et al., 1982, 1980; Mostafa et al., 1999), or human papillomavirus co-infection, particularly given potential associations with *p53* (Cooper et al., 1997; Helal et al., 2006; Khaled et al., 2003). These purported risk factors, however, remain controversial, given contradictory studies which dismiss potential interactions between smoking and schistosomiasis in terms of conferring increased cancer risk (Bedwani et al., 1997).

We speculate that alterations of *p53* expression in the urothelium during chronic urogenital schistosomiasis may set the stage for a vicious cycle of exacerbated bladder injury (caused by urothelial ulceration), inflammation, and wound repair responses that ultimately promote a pro-carcinogenic microenvironment. Further studies combining our ‘two-hit’ genetic lesion and chronic inflammation model with nitrosamine administration may help to characterize the early molecular and pathological events that lead to schistosomal bladder cancer.

Conclusions

Using an animal model of urogenital schistosomiasis, we have selectively altered activity of *p53*, a canonical tumor suppressor gene, in the urothelium to model early events in the transformation process during development of schistosomal bladder pre-neoplasia. This study indicates a possible sex difference in the changes observed due to this inflammatory insult in the context of *p53* haploinsufficiency. Using a similar approach, the role of alterations in other genes in the progression to schistosomal bladder cancer could be modeled to identify new means of early diagnosis and treatment.

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- Bladder urothelial ulceration may be precancerous in urogenital schistosomiasis (UGS)
- p53 defects have been found in schistosomal and non-schistosomal bladder cancers
- Women may have poorer early oncologic outcomes from bladder cancer
- p53 haploinsufficiency drives urothelial ulceration in female mice with UGS
- p53 defects and host sex may interact to determine UGS bladder cancer outcomes

Figure 1.

Tamoxifen-inducible Cre recombinase activity driven by uroplakin 3a results in urothelial-specific gene expression. A, fluorescent labeling of tamoxifen-inducible Cre recombinase activity in uroplakin3a (Upk3a)-expressing mouse bladder urothelial cells. We bred Upk3aCreERT2 mice to TdTomato-EGFP reporter mice to generate Upk3aCreERT2/wild type; TdTomato/wild type F1 mice (i.e., heterozygous at both loci of interest). Administration of 3 successive daily doses of tamoxifen induces Cre expression in Upk3a-positive cells that excise the floxed TdTomato gene, switching cells to EGFP expression.

Umbrella cells and intermediate cells are EGFP+. Some, but not all, basal cells of the urothelium become are EGFP+: see 10x magnification. * indicates bladder lumen. Frozen tissue, 5µm sections. Native fluorescence demonstrated in micrograph.

B, schematic demonstrating experimental groups of mice. Upk3a-GCE mice were crossed to p53-floxed mice and male and female F1 progeny were either injected with tamoxifen (“Tm” under “Cre activity” column) or corn oil vehicle 5, 6, and 7 days (triangles on timeline) prior to bladder wall injection with hamster tissue-derived *S. haematobium* eggs (“Egg” under “Bladder Injection” column), or hamster tissue control vehicle (“Hamster Extr” under “Bladder Injection” column). Tamoxifen injection rendered F1 mice haploinsufficient for *p53* (“insufficient” under “*p53*” column), whereas corn oil vehicle-injected F1 mice retained both wild type alleles of *p53* (“normal” under “*p53*” column).

Figure 2. Male mouse bladders injected with *Schistosoma haematobium* eggs demonstrate similar histologic features regardless of p53 sufficiency. Left panel, tamoxifen treated, hamster extract injected bladder from a male *Upk3a-GCE^{+/WT};p53^{flx/WT}* mouse (10X). Middle panel, male wild type p53 bladder treated with corn oil vehicle and injected with eggs and exhibiting urothelial hyperplasia and underlying granulomatous inflammation (25X). Right panel, male p53-haploinsufficient bladder injected with eggs exhibiting urothelial hyperplasia and underlying granulomatous inflammation (25X). H&E stained 5µm FFPE sections.

Figure 3.

Female p53-haploinsufficient mice exhibit urothelial ulceration and squamous metaplasia following bladder injection with *Schistosoma haematobium* eggs. Top panel, areas of ulceration and inflammatory cell infiltration overlying the granuloma (25X). Bottom panel, squamous metaplasia (25X). Female *Upk3a-GCE*^{+/WT}; *p53*^{flox}/WT mice were rendered p53-haploinsufficient by tamoxifen administration.

Tm + egg

Tm + egg

Figure 4.

Female p53-haploinsufficient mice exhibit dysplastic and other potentially preneoplastic changes of the urothelium following bladder injection with *Schistosoma haematobium* eggs. Top panel, egg-injected, p53-haploinsufficient female mouse bladder demonstrating nuclear dysplasia (large hyperchromatic nuclei and increased nucleocytoplasmic ratio, indicated by arrowheads, 25X). Bottom panel, egg-injected p53-haploinsufficient female mouse bladder with pre-invasive lesion, as evidenced by loss of normal urothelial cell polarity and orientation, and nuclear pleomorphism (25X).

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Table 1

Summary of bladder histology. Each pie chart within table cells portrays the proportion of mice in a given experimental group (rows) exhibiting the corresponding histologic finding (columns).

		Urothelium overlying granuloma								Urothelium distal to granuloma					
		normal	hyperplastic	hyperplastic with atypia	dysplastic (focal)	ulceration	infiltration by inflammatory cells	normal	hyperplastic	hyperplastic with atypia	dysplastic (focal)	ulceration	infiltration by inflammatory cells	squamous metaplasia	
Tm + Egg	♂														
	♀														
Veh + Egg	♂														
	♀														
Tm + Hamster	♂														
	♀														